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(71) Applicant: NOVAVAX, INC. [US/US]; 12111 Parklawn Drive, Rockville, MD 20852 (US).

(72) Inventor: WRIGHT, Craig, D.; 14740 Main Cove Terrace, Gaithersburg, MD 20878 (US).

(74) Agents: LOREN, Ralph, A. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

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(54) Title: GRAM NEGATIVE BACTERIAL INFECTION VACCINE

(57) Abstract

The present invention concerns an oral preparation useful as an immunizing agent or vaccine against gram negative bacterial infection. This oral preparation can also be used as a treatment for those infected with gram negative bacteria. The preparations can be used against any gram negative bacterial infection, including *Escherichia coli, Shigella flexneri 2a, and Salmonella enteriditis.*

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GRAM NEGATIVE BACTERIAL INFECTION VACCINE

BACKGROUND OF THE INVENTION

The present invention relates to oral vaccines. More particularly, the present invention concerns an oral vaccine, and methods for its use, for preventing and treating gram negative bacterial infection, e.g., *E. coli* infection. The vaccine uses a masking agent which disguises the fecal-like smell of the bacteria and allows oral use. The preferred masking agent also appears to provide adjuvant action.

Gram negative bacteria produce lipopolysaccharides as part of their cell walls. These lipopolysaccharides, which are designated as "smooth" or "rough" depending on their folding and other surface characteristics, have several regions, such as the O-side chain in the smooth isolates. The O-side chains of the various smooth lipopoysaccharides can act as epitopes for antibody production. While some work has been carried out attempting to isolate and purify these lipopolysaccharides as antigens for immunization purpose, this has not been very successful.

In 1977, Knowalchuk identified *Escherichia coli* isolates which produced a cytotoxin for Vero cells. Patients subsequently found to be infected with verocytotoxin-producing enterohemorrhagic *E. coli* (EHEC) strains (0157:H7, 026 or 0111) developed one of the following: an asymptomatic infection with toxin in the stool, diarrhea, or hemorrhagic colitis. *E. coli* 0157:H7 was the first EHEC strain identified in humans and remains the most common infectious cause of bloody diarrhea and hemorrhagic colitis in humans. Fifteen to thirty-seven per cent of patients presenting with bloody diarrhea are infected with *E. coli* 0157:H7. *E. coli* 0157:H7 is a gram negative sorbitol non-fermenter and can be identified in stool specimens from patients by plating stool on MacConkey media containing sorbitol rather than lactose. The definitive identification of the serotype is accomplished by utilization of a latex agglutination assay.

E. coli 0157:H7 outbreaks have been associated with inadequately cooked hamburger, cold meat, non-chlorinated drinking water, and close contact with colonized or infected persons in institutional settings (i.e., mental hospitals, nursing homes or daycare). Beef and dairy cattle, pigs, lambs and poultry may all be environmental reservoirs for verocytotoxin-producing enterohemorrhagic *E. coli*. A percentage of symptomatic *E. coli* 0157:H7-infected children and adults may subsequently develop Hemolytic-Uremic Syndrome (HUS) or Thrombotic Thrombocytopenic Purpura (TTP). The development of HUS or TTP is felt to be secondary to the production of Shiga-like verocytotoxins (SLT-1 and SLT-2) by EHEC. Verocytotoxins produced by EHEC strains inhibit protein synthesis at the level of elongation factor 1-dependent aminoacyl-tRNA binding to ribosomes.

Those patients with *E. coli* infections that develop HUS or hemorrhagic colitis have been shown by enzyme-linked immunosorbent assay (ELISA) and/or western blot to generate a convalescent serum antibody to lipopolysaccharide in 64-73% of patients.

Some earlier work has shown that murine monoclonal antibodies directed against the O-side chain epitopes of lipopolysaccharides from *Escherichia coli* and *Pseudomonas aeruginosa* serotypes are protective against gram negative infection when given i.p. in murine and rat sepsis models. *See*, e.g., Kaufman et al, "Monoclonal antibodies reactive with K-1 encapsulated *Escherichia coli* lipopolysaccharide are opsonic and protect mice against lethal challenge," Inf. and Imm. 52 (2):617-619 (1986); and Kim et al., "Functional activities of monoclonal antibodies to the O side chain of *Escherichia coli* lipopolysaccharides *in vitro* and *in vivo*," J. Infectious Disease 2:47-53(1988). However, no one has determined whether antibodies to O-side chain specific epitopes of *E. coli* 0157:H7, or any other EHEC, might be generated by oral vaccination with an inactivated *E. coli* 0157:H7 (or similar gram negative bacteria such as *Shigella flexneri* or *Salmonella enteriditis*) vaccine. One bar to administration of an oral vaccine has been the unpleasant, fecal-like smell associated with these bacteria.

Accordingly, it is an object of the invention to provide an oral vaccine to produce antibodies to O-side chain epitopes of lipopolysaccharides of gram negative bacteria, particularly against *E. coli, S. flexneri,* and *S. enteriditis.*

An additional object of the invention is to provide an oral vaccine against gram negative bacterial infection in which the fecal smell associated with the bacteria has been masked so there is a pleasing smell.

Another object of the invention is to provide oral vaccines against gram negative bacterial infection stable at -20°C and 4°C.

A further object of the invention is to provide a method of generating a serum antibody response to *E. coli, S. flexneri,* or *S. enteriditis* infection *in vivo.*

These and other objects and features of the invention will be apparent from the detailed description and the claims.

SUMMARY OF THE INVENTION

The present invention features an oral preparation for inoculation against gram negative bacteria, a method of preventing gram negative bacterial infection *in vivo* and a method of minimizing the effects of gram negative bacterial infection. The invention is particularly effective against the gram negative bacteria *E. coli, S. flexneri,* and *S. enteriditis.* The invention uses a combination product containing inactivated gram negative bacteria which contain the lipopolysaccharide antigen ("LPS"), and a masking agent. The masking agent is important because the gram negative bacteria such as *E. coli* still retain a fecal matter-like smell even after inactivation and/or lyophilization.

The basic preparation of the invention is an oral vaccine or medicament which provides protection against gram negative bacterial infection. This oral preparation includes the whole gram negative bacteria, in inactivated form, and a lipid vesicle-encapsulated masking agent. The preferred lipid vesicles for use in the invention are non-phospholipid, paucilamellar lipid vesicles having 2-8 lipid bilayers surrounding an amorphous central cavity. The preferred masking agent is a fragrance or flavoring encapsulated in the amorphous cavity of the lipid vesicles, most preferably in the form of a water-immiscible material which substantially fills the amorphous central cavity. Particularly preferred materials are the volatile oils such as cherry or peppermint oil. While the invention could be used to prevent any gram negative bacterial infection, verocytotoxin-producing enterohemorrhagic *E. coli* cells, *S. flexneri* cells, and *S. enteriditis* were selected. However, the method would also be effective using other gram negative bacteria, including bacteria of the families *Spirosomaceae, Pseudomonadaceae, Azotobacteraceae, Rhizobiaceae, Methylococcaceae, Halobacteriaceae, Acetobacteraceae, Legionallaceae, Neisseriaceae, Vibrionaceae, Pasteurellaceae, Bacteroidaceae,* and *Enterobacteriaceae,* particulary the genera *Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Erwinia, Serratia, Hafnia, Edwardsiella, Proteus, Providencia, Morganella* and *Yersinia.* Other genera include *Aquaspirillum, Spirillum, Azospirillum, Oceanospirillum, Campylobacter, Helicobacter, Bdellovibrio, Vampirovibrio,* and *Gardinella.* Whatever gram negative bacteria is used, the cells are inactivated using a standard method such as heat or formalin inactivation. The cells can be lyophilized and reconstituted with a reconstituting solution before use or they can be used immediately as is. If they are not lyophilized, the activity can deteriorate rapidly but if they are lyophilized and reconstituted, the lipid vesicle encapsulated masking agent can be used as the reconstituting solution.

The methods of the invention can be used to provide protection against gram negative infection, e.g., acting as a vaccine. An effective amount of the oral gram negative bacterial

preparation is used to generate antibodies *in vivo*. The preferred antigens for generating the antibodies are the O-side chain epitopes of the lipopolysaccharides on the surfaces of strains of *E. coli* 0157:H7, *S. flexneri 2a*, and *S. enteriditis* bacteria. Since other gram negative bacteria have similar O-side chains on their lipopolysaccharides, similar procedures can be used for such other bacteria, as is known in the art. The bacteria are grown and isolated using standard microbiological methods and inactivated by heat or formalin. The resulting inactivated cells can be lyophilized using standard techniques and reconstituted. The oral product described previously is the preferred agent for providing protection in the form of a vaccine or for use in prevention of infection by generating antibodies to help minimize the effects of the infection.

Further advantages and details of the invention will be apparent from the detailed description and the claims.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows murine IgM levels at day 58 after vaccination with *E. coli* 0157:H7 vaccine;

Figure 2 shows murine IgG levels at day 58 after vaccination *E. coli* 0157:H7 vaccine;

Figure 3 shows antibody titers after a 7.5 mg dose of the *E. coli* 0157:H7 vaccine;

Figure 4 shows antibody titers after a 1.875 mg dose of the *E. coli* 0157:H7 vaccine;

Figure 5 shows antibody titers after a 2.618 mg dose of the *S. flexneri 2a* vaccine;

Figure 6 shows antibody titers after a 10.47 mg dose of the *S. flexneri 2a* vaccine;

Figure 7 shows antibody titer after a 3.937 mg dose of the *S. enteriditis* vaccine; and

Figure 8 shows antibody titers after a 15.89 mg dose of the *S. enteriditis* vaccine.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an oral preparation useful in vaccination against gram negative bacterial infection and method of its use. The same general preparation can also be used to treat gram negative bacterial infection by increasing the *in vivo* antibody response.

The basic process for producing the oral preparation of the invention commences with growth and harvest of the bacteria, inactivation, preferably but not exclusively with formaldehyde, and lyophilization of the whole cells. This process appears to maintain the antigenic integrity of the bacteria. Because of the feculent nature of the cells, the preferred diluent contains an aromatic oil, preferably a peppermint oil or a cherry-flavored oil, encapsulated in a non-phospholipid liposome (Novasome®) to enhance the palatability of the vaccine. These flavored Novasomes are composed of glycerol monostearate, soya sterols, soybean oil, cherry or peppermint oil, polysorbate 60, oleic acid, and water for injection. Details for preparation of lipid vesicles containing oil are disclosed in United States Patent No. 4,911,928, the disclosure of which is incorporated herein by reference. However, other materials constituting the lipid vesicles, and other production methods, could be used so long as the flavor masking provision is met. An additional advantage of using the lipid vesicles described herein is that these vesicles appear to provide adjuvant activity in addition to their flavor masking capability. While such adjuvant activity is not necessary for practice of the invention, it may raise antibody titers, as described in U.S. Serial No. 08/201,346, entitled "Vaccines Containing Paucilamellar Lipid Vesicles as Immunological Adjuvants", incorporated herein by reference.

The following example shows a lyophilized, formalin-inactivated *E. coli* 0157:H7 oral vaccine, reconstituted with cherry-flavored lipid vesicles in water for injection, which has been tested in animal models for both safety and immunogenicity. The vaccine is safe and immunogenic in mice and rats using a two-dose gavage regimen.

EXAMPLE I

This example illustrates the steps in preparing an oral vaccine of the invention, as well as tests for its safety. The *E. coli* 0157:H7 vaccine described herein is a sterile, formalin-inactivated whole bacteria, lyophilized product in a single-use vial containing 300 mg bacterial protein in phosphate-buffered saline, pH 7.5. The vaccine is stored at -20°C until reconstitution with 10 mL of the Novasome-WFI diluent. The reconstituted vaccine should be administered within one hour.

The Novasome-WFI diluent is a paucilamellar, non-phospholipid liposome containing cherry-flavored oil to enhance the palatability of the *E. coli* 0157:H7 vaccine. This Novasome preparation is composed of glycerol monostearate (7.9%), soya sterols (2.2%), soybean oil (9.2%), cherry oil (4.5%), polysorbate 60 (2.1%), oleic acid (0.1%) and water for injection (74%). After production of the Novasome lipid vesicles, they are diluted with WFI in a ratio of Novasomes:WFI of 1:32 (v/v). The final percentage of water in the Novasome-WFI diluent is 99.2%. The resultant Novasome-WFI diluent is a sterile Novasome suspension in water for injection in a single use vial. The Novasome-WFI diluent is stored at

room temperature. Ten milliliters of the Novasome-WFI diluent are utilized to reconstitute each bottle of the *E. coli* 0157:H7 vaccine.

The following steps are followed to produce the 0157:H7 vaccine.

Preparation of NOVASOME-WFI Diluent Vehicle

Step 1. Preparation of Lipid Oil Phase

The lipid phase consisted of:

• Glycerol Monostearate	13.72g
• Polysorbate 60 (Tween 60)	3.64g
• Generol 122 (Refined Soya Sterols)	3.84g
• Oleic Acid	240µl
• Cherry Oil	8.0g
• Soybean Oil	16.0g

All raw materials were added to a depyrogenated vessel which was then heated on a heating plate with agitation until all materials were melted. The lipid solution was then transferred to a sterile 50 mL conical centrifuge tube which was placed in an 85°C circulating water bath for one hour.

Step 2. Preparation of Diluent

The diluent phase consisted of:

3.7 mL sterile water for injection for each 5 mL of Novasome preparation.

For each preparation, the sterile water for injection was drawn up in a 5cc syringe which was placed in an incubator and heated to 65°C.

Step 3. Preparation of Mixing Instrument

A syringe mixing machine using 5cc syringes was set for a lipid/oil to diluent ratio of 1.3 : 3.7. The speed controlling the force of pressure for mixing was adjusted to 70. This instrument is described in United States Patent No. 4,895,452, the disclosure of which is incorporated herein by reference.

Step 4. Preparation of Novasome Lipid Vesicles

The heated lipid was drawn into a 5cc LuerLok syringe and connected to the diluent syringe by way of a two-way stainless steel connector.

The syringes were then positioned into the Teflon mixing block and placed on the mixing machine. The solutions in the syringes were allowed to mix by pushing the liquids

back and forth from one syringe to the other under pressure for 10 strokes at room temperature and then rapidly cooled using CO_2 for 90 strokes.

Several 5cc Novasome lipid vesicle preparations were combined into a single solution in a depyrogenated vessel. Samples of the final pooled Novasome preparation were taken for USP sterility testing, endotoxin testing, pH determination, stability testing, and sub-micron particle sizing. More particularly, USP Sterility testing was performed on a 1.0mL sample of the pooled Novasome preparation. No bacterial growth was observed in the liquid medium.

Step 5. Dilution of Novasomes

4,850 mL of sterile water for injection was filtered through a 0.2μm filtering unit into a depyrogenated vessel containing a stir bar. 150 mL of pooled Novasome preparations was aseptically added to the diluent. The suspension was placed on a stir plate and allowed to mix for 24 hours. Three 5L bottles of Novasome-WFI diluent were prepared. Samples were taken from each 5L Novasome-WFI diluent bottle for USP sterility testing, endotoxin testing, and sub-micron particle sizing.

A 10μL sample of the vaccine was plated onto TSA which was incubated for 48 hours at 37°C as a test for sterility of the larger batch of diluent. Again, there was no growth observed on the solid or liquid medium.

E. coli 0157:H7 Vaccine Manufacturing and Control Data

Preparation of *E. coli* 0157:H7 Active Substance

Step 1. Seed Culture

The *Escherichia coli* 0157:H7 active material was derived from the *Escherichia coli* strain 0175:H7 (ATCC 43894). The seed was stored in 15% Glycerol Trypticase Soy broth at -70°C and identified prior to initiating the starter culture. Culture identification was established by incubating the culture overnight on Trypticase Soy Agar with 5% Sheep Blood and MacConkey's Agar. The culture showed a homogenous smooth colonial morphology and revealed only gram negative rods by gram stain. The API biochemical profile was consistent with *E. coli* 0157:H7.

Step 2. Starter Culture Tubes

One colony from the TSA II plate of the seed culture was used to inoculate each of two 15 mL tubes of Trypticase Soy Broth (BBL). The cultures were grown on a rotor for 2 hours at 37°C, after which a subculture was taken for identity check.

Step 3. Starter Culture Flask

12 mL from the Trypticase Soy Broth culture was used to inoculate a Fernbach flask containing 1L of Lauria Broth medium. The culture was grown on an incubator shaker at 150 rpm 37°C for 3.5 hours after which a subculture was taken for identity check. The culture was incubated overnight on Trypticase Soy Agar with 5% Sheep Blood and MacConkey's Agar. The culture showed a homogenous smooth colonial morphology and revealed only gram negative rods by gram stain. The API biochemical profile was consistent with *E. coli* 0157:H7.

Step 4. Preparation of Growth and Fermentation Medium

Lauria Broth medium (pH 7.0) was used for the starter culture flask, as well as the fermentation process. The medium consisted of:

- Tryptone 20g/L
- Yeast Extract 10g/L
- Sodium chloride 10g/L

The starter flask medium was sterilized in a steam autoclave for 30 minutes at a temperature setting of 121°C. The fermentation medium was sterilized in the fermentor at a temperature setting of 121°C for 30 minutes. Samples of both media were taken for sterility testing prior to initiating the starter culture or the fermentation.

Step 5. Fermentation

Deionized water was added to a clean batch vessel. The ingredients for Lauria Broth medium were added with sufficient agitation for mixing and sufficient additional deionized water was then added to obtain 40L. The solution was mixed, pumped into a 50L B. Braun Fermento (Model Biostat U-50) and chased with sufficient deionized water to achieve a 50L batch volume. The unit was sealed and the sterilization cycle started. 4L of a 40g/L dextrose solution was prepared and sterilized using a 0.2µm membrane filter. The feed transfer line was aseptically connected to the fermentor and the dextrose feed was added. The incubation conditions (pH 7.0 +/- .02, temperature 37°C +/-1°C, dissolved oxygen (D.O.) .+/-50%, air flow 80 +/-5 SLPM, agitation D.O. cascade, 300 rpm/min.) were automatically maintained. The pH was maintained with an automatic controller utilizing 20% glacial acetic acid and 4N sodium hydroxide. Foaming was automatically controlled utilizing PPG 2000 in an addition vessel. The 1L starter culture was used to inoculate the fermentor.

The *E. coli* bacteria cells were allowed to ferment into log phase for 10 hours. Samples were obtained hourly for optical density (OD) measurement. When absorbance approached 1.0, dilutions were performed. Dextrose usage and foaming were monitored. Subcultures were taken from the starter flask and at the end of the fermentation run for identity testing of the isolate . Bacterial count (CFU/mL), pH, dextrose analysis and OD were performed on the final product.

Step 6. Harvest

Cells were harvested using a Heraeus Varifuge (Model 20 RS) continuous centrifuge for 5.25 hours. Speed of the continuous rotor was controlled at 15,000 rpm and temperature was controlled to less than 25°C. Supernatant samples were analyzed periodically during centrifugation for optimization of centrifugation. 250g of cell paste was then aseptically transferred to each of 5 1L Nalgene centrifuge bottles for further processing. Centrifuge bottles were weighed prior to addition of cell paste and the weight was recorded.

Step 7. Formaldehyde Treatment of Cell Paste

Six liters of 1.5% formaldehyde solution were prepared using Phosphate Buffered Saline (PBS) (146mM Na-10mM PO_4) diluent. Samples of PBS were taken for USP Sterility and endotoxin testing as well as pH determination. The 1.5% Formaldehyde PBS solution was added to each centrifuge bottle to achieve a weight of 1,035g. The paste was resuspended by agitation after which centrifuge bottles were allowed to stir at 4°C on a stir plate for 96 hours. Samples from each centrifuge bottle were taken at 24 and 96 hours and plated on TSA II plates for monitoring of viability.

After 96 hours, the formaldehyde inactivated paste was centrifuged for 4 hours at 4000 rpm 4°C in a Beckman refrigerated centrifuge (Model J-6B). Supernatant was discarded and sufficient PBS was added to each bottle to achieve the initial weight. Paste was resuspended by agitation, after which bottles were centrifuged for 4 hours at 4000 rpm at 4°C in a Beckman refrigerated centrifuge (Model J-6B). The supernatant from this centrifugation was discarded and the wash process was repeated. Samples of PBS were taken for USP Sterility and endotoxin testing (see IPC) as well as pH determination. There was no growth observed in the liquid medium. An endotoxin assay was also performed using the Limulus Lysate Assay (Cape Cod Associates). The endotoxin level was at an acceptable level of <.03 EU/mL. As an additional check on the viability, a 10µL sample of the product was taken at 24 and 96 hours after formalin inactivation and plated onto TSA II which was incubated for 48 hours at 37°C. There was no growth observed on the solid medium.

Step 8. Resuspension of Formaldehyde Inactivated Paste

The formaldehyde inactivated bacterial paste was resuspended by agitation in PBS at a concentration of 1g/5mL of PBS. Samples were taken from each centrifuge bottle of the final product for USP sterility testing. USP Sterility testing was performed on a 1.0mL sample of the resuspended formaldehyde inactivated paste. A 10µL sample of the vaccine was also plated onto TSA which was incubated for 48 hours at 37°C. There was no growth observed on the solid or liquid medium.

Step 9. Lyophilization

The formaldehyde inactivated vaccine was aseptically filled into 50mL vials using a Sepco filling machine with presterilized syringe and tubing apparatus, with 10mL of the paste placed in each vial. The product was frozen to -40°C on the lyophilizer shelf. The lyophilization chamber was evacuated to 100µm and the product remained at -20°C for 16 hours. The temperature was then elevated to 0°C for 7 hours, after which it was elevated to 25°C for 14 hours. 40 samples were taken for USP Sterility testing and 10 samples were analyzed for residual moisture assay. The vaccine passed both tests. The lyophilized vaccine was stored at -20°C.

Murine Immunogenicity Study

Mice were divided into 4 groups of 10 mice each. All the mice in a group received the *E. coli* 0157:H7 vaccine reconstituted in cherry-flavored Novasome-WFI diluent at doses of 7.50, 1.875, 0.9375, and 0.1875 mg of protein, respectively. Vaccine doses were given on Day 0 and Day 30 and were administered by gavage. The animals were prebled 4 days before receiving their first vaccine dose, and were test-bled on Days 14, 28, 44, and 58.

Two animals died during the course of the study: one in the 1.875 mg dose group died on Day 30, and one in the 0.9375 mg dose group died on Day 5.

Mouse sera were tested by ELISA for the presence of antibody to *E. coli* 0157:H7 LPS. Test sera were serially diluted in flat-bottomed ELISA plates previously coated with *E. coli* 0157:H7 LPS and blocked. After two hours of incubation at room temperature, the plates were washed and incubated with enzyme-labeled goat anti-mouse IgM or anti-mouse IgG second antibody for 1 hour. After further washing, the plates were developed with a colorigenic substrate, and read using an automated ELISA plate reader. Anti-LPS antibody titers were the reciprocal of the serum dilution producing an optical density (OD) reading 3X the OD reading of the homologous prebleed.

The IgG subclass response was determined by using enzyme-labeled second antibodies specific for mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3.

Sera showing high pre-bleed ELISA titers against *E. coli* 0157:H7 LPS were screened for antibody specificity by western blot. Purified *E. coli* 0157:H7 LPS was separated by polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose sheets, which were then blocked, cut into strips, and dried. For screening, strips were incubated with a 1:100 dilution of the test serum for 2-4 hours, washed, incubated with an enzyme-labeled second antibody, rewashed, and developed with a precipitating colorigenic substrate.

Both the LPS-specific IgM and IgG titers at Day 58 were dose-dependent. The two higher doses of vaccine produced IgM titers on Day 58 that were roughly equivalent (Figure 1), as were the 58-day IgG titers at the same two doses (Figure 2). In both cases, the tiers resulting from the lower doses of vaccine were correspondingly lower. The 58-day IgG titers were approximately 10-fold higher than the IgM titers.

Significant IgM titers were obtained 14 days after the initial dose with the vaccine preparation at doses of 7.50 mg (Figure 3) and 1.875 mg (Figure 4). IgM displayed an anamnestic response after the second vaccine dose. IgG titers, which were low 28 days after the initial dose, rose dramatically after the second dose, and remained high at Day 58 (28 days after the second dose; Figures 3 and 4).

EXAMPLE II

This example illustrates a second oral vaccine of the invention. Like the *E. coli* preparation of Example I, this vaccine is also prepared using sterile formalin-inactivated whole bacteria. In this case, the bacteria is *Shigella flexneri 2a*. The vaccine, 419 mg bacterial protein in PBS at pH 7.15, is stored at -20°C until reconstituted with 10 mL of the Novasome WFI diluent.

The diluent and the vaccine are prepared according to the procedures set forth in Example I.

Murine Immunogenicity Study

Mice were divided into four groups of 15 mice each. The mice in each group received the *S. flexneri 2a* vaccine reconstituted in cherry flavored Novasome diluent at doses of 10.47 mg, 2.618 mg, 1.309 mg, and 0.2618 mg of protein, respectively. Vaccine doses were given on Day 0 and Day 30, and were administered by gavage. The animals were bled 4 days before receiving their first vaccine dose, and were test-bled on Days 14, 28, 44 and 58.

Sera showing high pre-bleed ELISA titers against *S. flexneri* LPS were screened for antibody specificity by western blot. Purified *S. flexneri* LPS was separated by

polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose sheets, which were then blocked, cut into strips, and dried. For screening, strips were incubated with a 1:100 dilution of the test serum for 2-4 hours, washed, incubated with an enzyme-labeled second antibody, rewashed, and developed with a precipitating colorigenic substrate.

Both the LPS-specific IgM and IgG titers at Day 58 were dose-dependent. IgG and IgM titers rose dramatically after the second dose, and remained high at Day 58 (28 days after the second dose; Figures 5 and 6).

EXAMPLE III

This example illustrates a third oral vaccine of the invention. Like the *E. coli* preparation in Example I, this vaccine is also prepared using sterile formalin-inactivated whole bacteria. In this case, the bacteria is *Salmonella enteriditis*. The vaccine, 653 mg bacterial protein in PBS at pH 7.15, is stored at -20°C until reconstituted with 10 mL of the Novasome WFI diluent.

The diluent and the vaccine are prepared according to the procedures set forth in Example I.

Murine Immunogenicity Study

Mice were divided into four groups of 15 mice each. The mice in each group received the *Salmonella enteriditis* vaccine reconstituted in cherry flavored Novasome diluent at doses of 15.89 mg, 3,973 mg, 1.986 mg, and 0.397 mg of protein respectively. Vaccine doses were given on Day 0 and Day 30, and were administered by gavage. The animals were bled 3 days before receiving their first vaccine dose, and were test bled on Days 14, 28, 44, and 58.

Sera showing high pre-bleed ELISA titers against *S. enteriditis* LPS were screened for antibody specificity by western blot. Purified *S. enteriditis* LPS was separated by polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose sheets, which were then blocked, cut into strips, and dried. For screening, strips were incubated with a 1:100 dilution of the test serum for 2-4 hours, washed, incubated with an enzyme-labeled second antibody, rewashed, and developed with a precipitating colorigenic substrate.

Both the LPS-specific IgM and IgG titers at Day 58 were dose-dependent. IgG and IgM titers rose dramatically after the second dose, and remained high at Day 58 (28 days after the second dose; Figures 5 and 6).

From the foregoing, it is apparent that the orally administered, inactivated whole virus vaccines of the present invention are safe and immunogenic.

The foregoing description of the invention is meant to be only exemplary and is not intended to limit the scope of the invention. The invention is defined by the following claims.

What is claimed is:

CLAIMS

1. An oral vaccine preparation for generating anti-LPS antibodies for preventing gram negative infection, said oral vaccine preparation comprising inactivated gram negative bacteria cells and a lipid vesicle encapsulated flavor masking agent.

2. The oral vaccine of claim 1 wherein said lipid vesicles comprise paucilamellar lipid vesicles having 2-8 lipid bilayers surrounding an amorphous central cavity.

3. The oral vaccine of claim 2 wherein said paucilamellar lipid vesicles comprise non-phospholipid materials as the primary lipids in their bilayers.

4. The oral vaccine of claim 2 wherein said flavor masking agent comprises a fragrance encapsulated in said amorphous cavity of said lipid vesicles.

5. The oral vaccine of claim 4 wherein said fragrance comprises a water-immiscible material substantially filling said amorphous central cavity.

6. The oral vaccine of claim 5 wherein said fragrance comprises a flavored oil.

7. The oral vaccine of claim 1 wherein said gram negative bacteria is selected from the group consisting of *Spirosomaceae, Pseudomonadaceae, Azotobacteraceae, Rhizobiaceae, Methylococcaceae, Halobacteriaceae, Acetobacteraceae, Legionallaceae, Neisseriaceae, Vibrionaceae, Pasteurellaceae, Bacteroidaceae,* and *Enterobacteriaceae.*

8. The oral vaccine of claim 1 wherein said gram negative bacteria is selected from the group consisting of *Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Erwinia, Serratia, Hafnia, Edwardsiella, Proteus, Providencia, Morganella* and *Yersinia.*

9. The oral vaccine of claim 8 wherein said gram negative bacteria comprises enterohemorrhagic *E. coli* bacteria.

10. The oral vaccine of claim 8 wherein said *E. coli* cells are selected from the group consisting of 0157:H7. 026 and 0111 cells.

11. The oral vaccine of claim 8 wherein said gram negative bacteria comprises *Shigella flexneri 2a* bacteria.

12. The oral vaccine of claim 8 wherein said gram negative bacteria comprises *Salmonella enteriditis* bacteria.

13. The oral vaccine of claim 1 wherein said gram negative bacterial cells are selected from the group consisting of formalin inactivated and heat inactivated cells.

14. The oral vaccine of claim 1 wherein said gram negative bacterial cells are lyophilized and reconstituted with a reconstituting solution before use.

15. The oral vaccine of claim 1 wherein said lipid vesicle encapsulated flavor masking agent comprises a reconstituting solution.

16. A method of providing protection against gram negative bacterial infection *in vivo* comprising the step of oral administration of an effective amount of an oral vaccine including as active components inactivated gram negative bacterial cells and a lipid vesicle encapsulated flavor masking agent.

17. The method of claim 16 wherein said lipid vesicles comprise paucilamellar lipid vesicles having 2-8 lipid bilayers surrounding an amorphous central cavity.

18. The method of claim 17 wherein said paucilamellar lipid vesicles comprise non-phospholipid materials as the primary lipids in their bilayers.

19. The method of claim 17 wherein said flavor masking agent comprises a flavoring encapsulated in said amorphous cavity of said lipid vesicles.

20. The method of claim 19 wherein said flavoring comprises a water-immiscible material substantially filling said amorphous central cavity.

21. The method of claim 20 wherein said flavoring comprises a flavored oil.

22. The method of claim 16 wherein said gram negative bacteria is selected from the group consisting of *Spirosomaceae, Pseudomonadaceae, Azotobacteraceae, Rhizobiaceae, Methylococcaceae, Halobacteriaceae, Acetobacteraceae, Legionallaceae, Neisseriaceae, Vibrionaceae, Pasteurellaceae, Bacteroidaceae,* and *Enterobacteriaceae.*

23. The method of claim 16 wherein said gram negative bacteria is selected from the group consisting of *Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella,*

Enterobacter, Erwinia, Serratia, Hafnia, Edwardsiella, Proteus, Providencia, Morganella and Yersinia.

24. The method of claim 23 wherein said gram negative bacteria comprises *E. coli* cells.

25. The method of claim 24 wherein said verocytotoxin-producing *E. coli* cells are selected from the group consisting of 0157:H7, 026 and 0111 cells.

26. The method of claim 23 wherein said gram negative bacteria comprises *Shigella flexneri 2a* cells.

27. The method of claim 23 wherein said gram negative bacteria comprises *Salmonella enteriditis* cells.

28. The method of claim 16 wherein said gram negative bacterial cells are formalin inactivated.

29. The method of claim 16 wherein said gram negative bacterial cells are lyophilized and reconstituted with a reconstituting solution before use.

30. The method of claim 29 wherein said reconstituting solution comprises said lipid vesicle encapsulated flavor masking agent.

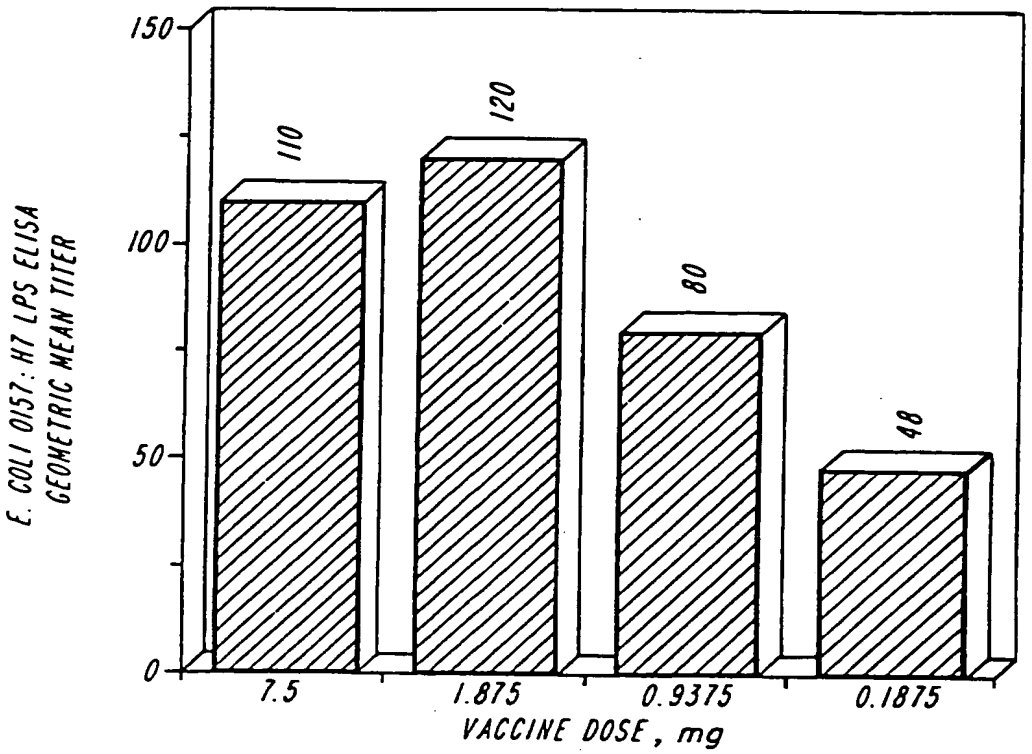

FIG. 1

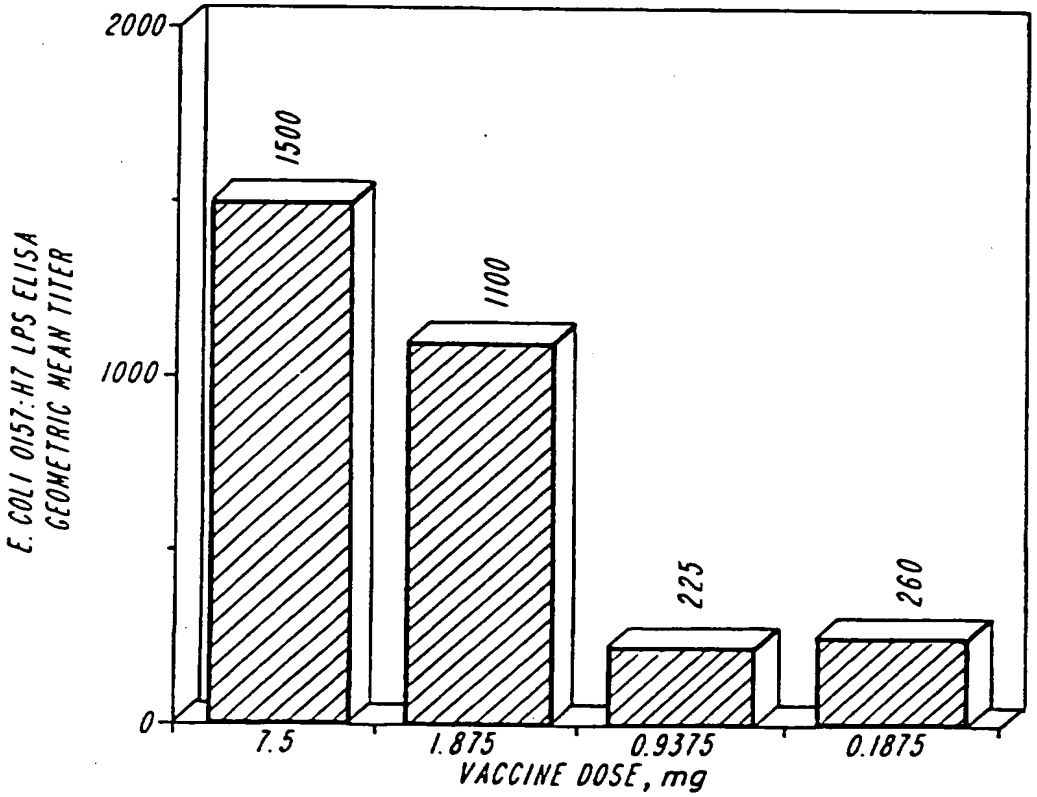

FIG. 2

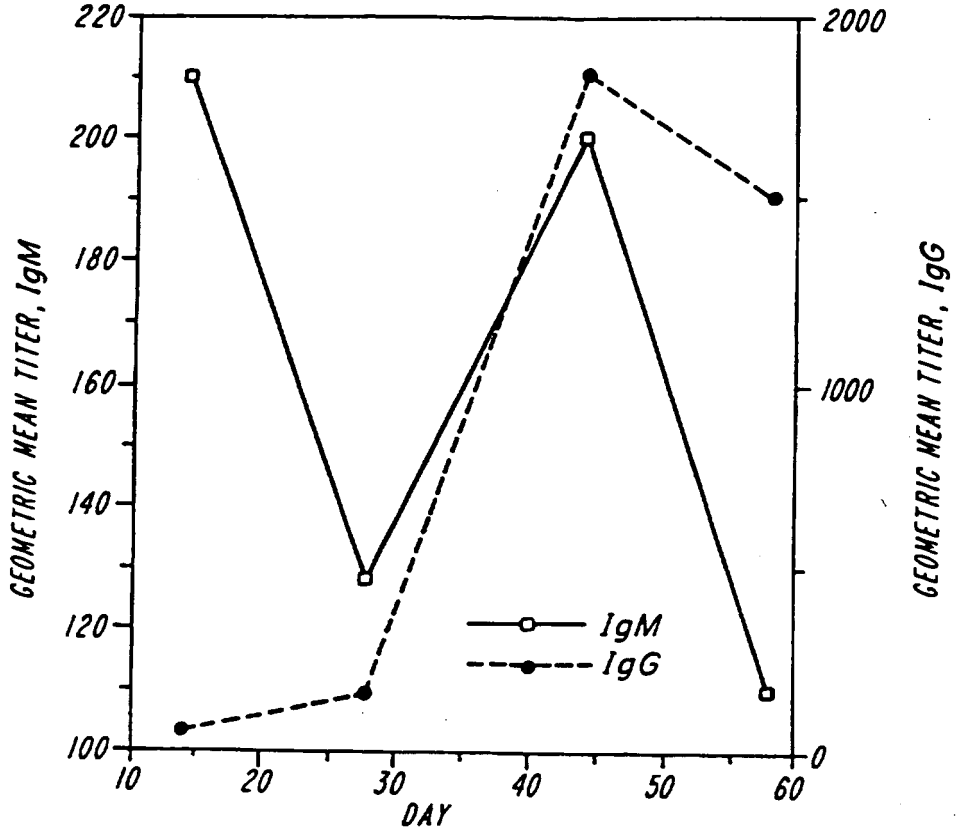

FIG. 3

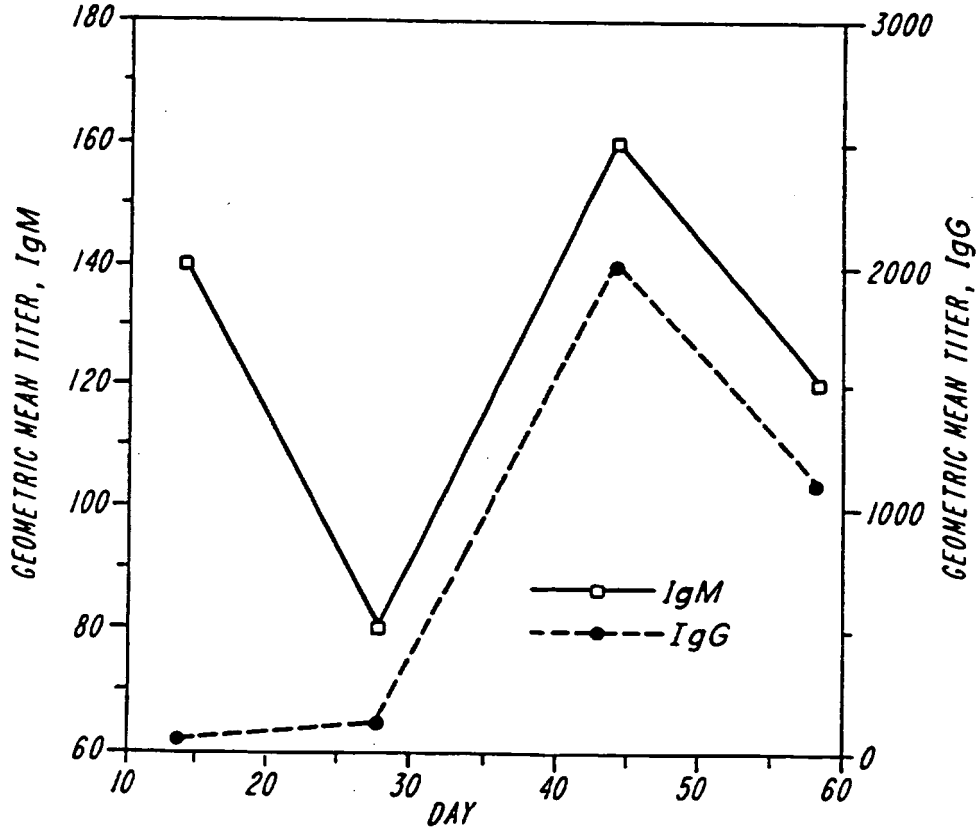

FIG. 4

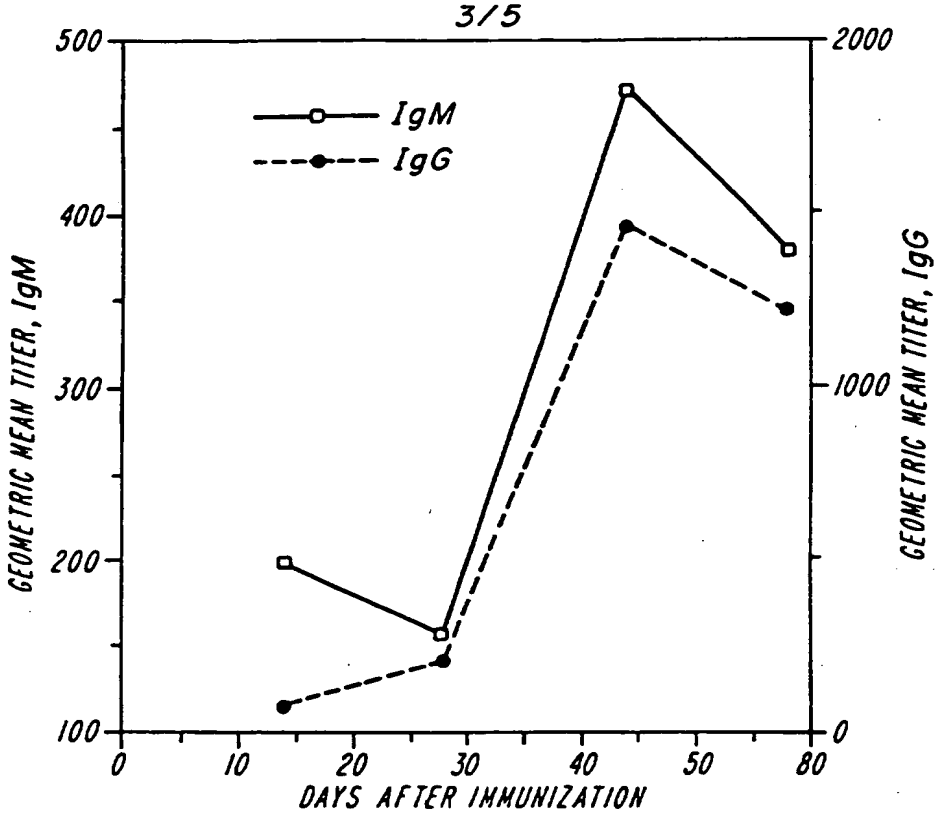

FIG. 5

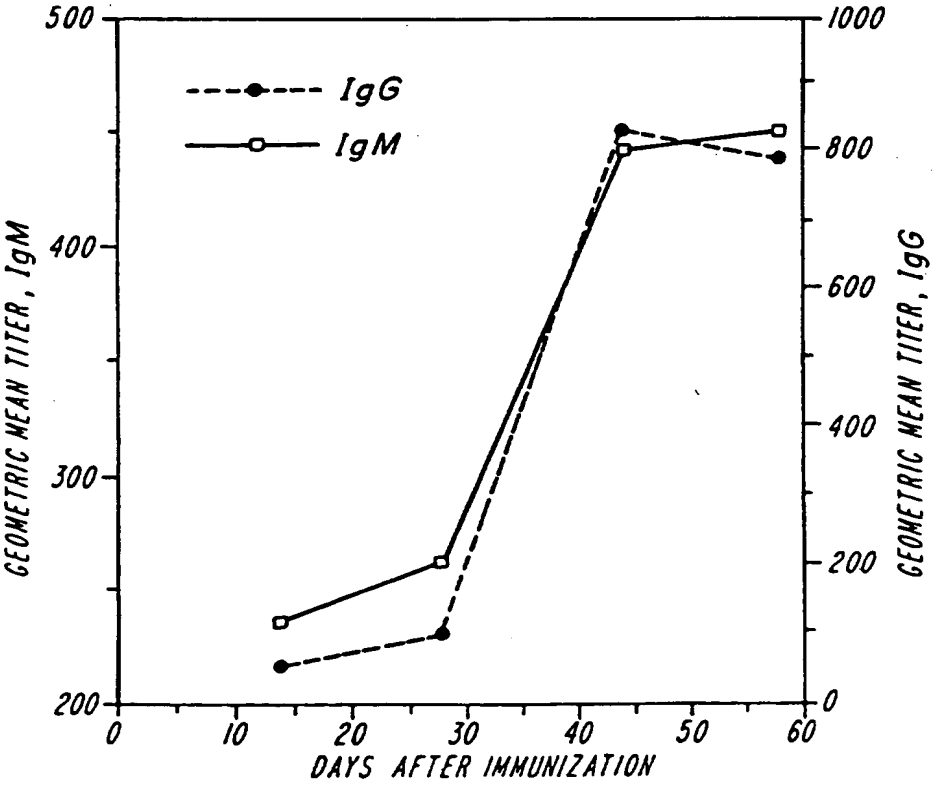

FIG. 6

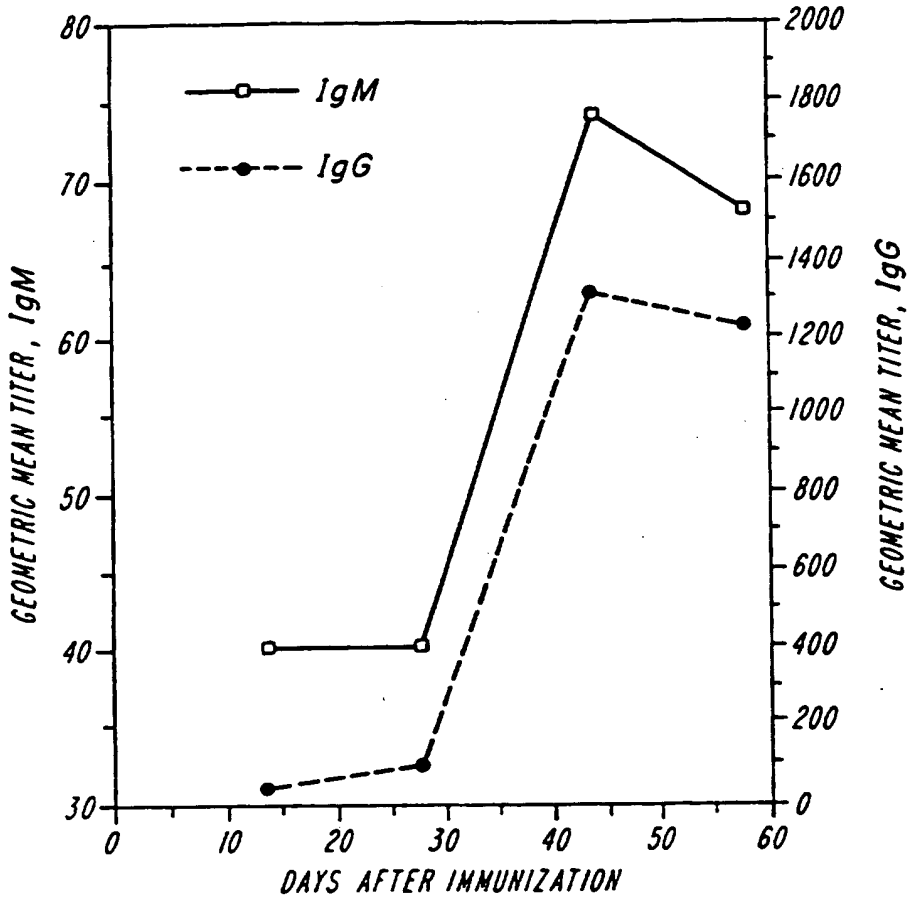

FIG. 7

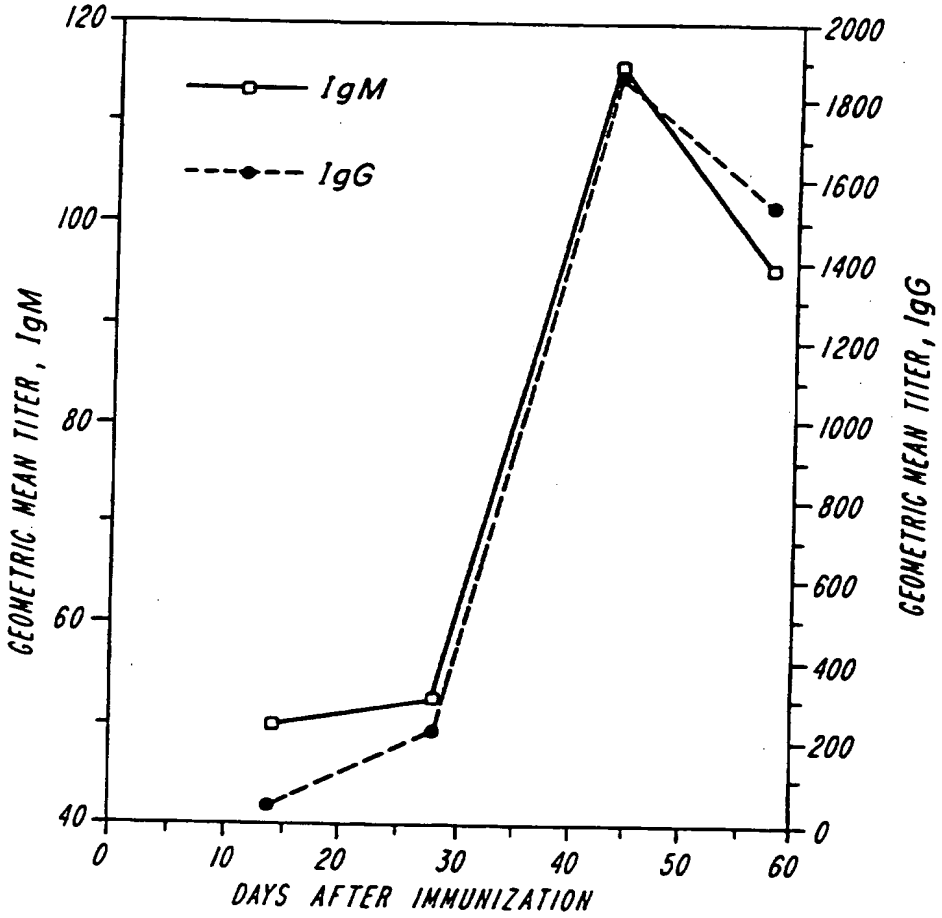

FIG. 8

INTERNATIONAL SEARCH REPORT

Int. ..ational application No.
PCT/US95/15446

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/184.1, 241.1, 257.1, 258.1, 417, 450
According to International Patent Classification (IPC) r to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 241.1, 257.1, 258.1, 417, 450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Scientific American, Volume 256, No.1, issued January 1987, Ostro, "Liposomes", pages 102-111, see page 110.	1-30
Y	Immunology Today, Volume 11, issued 1990, Gregoriadis, "Immunological Adjuvants: a role for liposomes", pages 89-97, see page 93.	1-30.
Y	US, A 5,009,819 (POPESCU ET AL.) 23 April 1991, see column 1.	1-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
11 MARCH 1996

Date of mailing of the international search report
20 MAR 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
H. F. SIDBERRY
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

Inte.. national application No.
PCT/US95/15446

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 9/127, 9/133, 39/00, 39/40, 39/095, 39/108, 39/112; A01N 25/26, 25/28